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PRINCIPAL INVESTIGATOR: Rayhana Rahim
Jeannine S. Strobl, Ph.D.

CONTRACTING ORGANIZATION: West Virginia University Research Corporation
Morgantown, WV 26506-6845

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Abstract

We have shown that the quinoline antimalarials chloroquine (CQ) and hydroxychloroquine (HCQ) inhibit proliferation and induce differentiation in breast cancer cell lines without toxicity to normal MCF-10A cells. The purpose of this project is to derive more efficacious antitumor agents that enhance the differentiation response by using CQ and HCQ in combination with the demethylating agent, 5-Aza-2'-deoxycytidine (5-Aza-dC; Aza), or with the differentiating agent, all-trans-Retinoic acid (ATRA). Cell survival, cellular differentiation, histone H3 and/or histone H4 acetylation status, and HDAC protein and activity were measured to show that combination of Aza or ATRA with the quinolines augmented the antiproliferative effect, differentiation response, and acetylation status of either CQ or HCQ alone. A new and highly sensitive assay for histone acetylation by mass spectrometry was developed to illustrate the specific lysine sites that get modified (acetylated/deacetylated) by the most promising combination of chemotherapeutic agents. This approach will be pivotal in further developing more effective and less toxic therapeutic agents for breast cancer intervention.

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Introduction:

An impeding challenge to breast cancer drug therapies is the availability of more effective and less toxic chemotherapeutic agents that do not relay harm to neighboring normal breast cells and tissues. Preliminary studies showed that the quinoline antimalarials, chloroquine (CQ) and hydroxychloroquine (HCQ), inhibit proliferation and induce differentiation in breast cancer cell lines without toxicity to normal MCF-10A breast cells. Hence, the goal of these studies was to explore: (1) whether a drug combination modulating epigenetic events would sensitize breast cancer cells to the antitumor activity of CQ or HCQ, (2) and if so, which would be the most promising combination of agents for the generation of safer and less toxic chemotherapeutic agents for the prevention and treatment of advanced breast cancer. We hypothesized that the use of a drug combination modulating epigenetic events would lower the concentration of CQ or HCQ needed to produce the differentiation response. Hence, the quinoline agents were used in combination with the demethylating agent, 5-Aza-2'-deoxycytidine (5-Aza-dC or Aza), or with the differentiating agent, all-trans-Retinoic acid (ATRA) in order to lower the threshold for chemotherapy-induced cell death in breast cancer cells. Cell survival, cellular differentiation, and histone H3/H4 acetylation status were measured to show that combination of 5-Aza-dC or ATRA with the quinolines sensitized breast cancer cells to enhanced growth inhibition, differentiation, and histone hyperacetylation compared to cells treated with either CQ or HCQ alone. Furthermore, ATRA was identified as a direct HDAC inhibitor and upon combination treatment with HCQ had the most significant inhibitory effect on tumor cell clonogenic survival in both estrogen receptor (ER+) and (ER-) breast cancers. Thus, ATRA combined with HCQ served as the most promising combination of chemotherapeutic agents and were subsequently assessed using a new and highly sensitive assay for histone acetylation by mass spectrometry to generate a profile on lysine modification (acetylation/deacetylation). The combination of ATRA with HCQ increased acetylation of N-terminal lysine residues on histones H3/H4 as well as caused direct HAT activation. The overall data suggest that HCQ might contribute to breast cancer cell differentiation by modulating HAT activity; whereas, ATRA sensitizes breast cancer cells to the antitumor activity of HCQ via regulation of both HAT and HDAC enzymatic activities.

Body:

Research Accomplishments

Task #1 of this project was to determine optimal conditions for cellular differentiation using CQ or HCQ ± 5-Aza-dC (Aza) or ATRA (or a vitamin D-derivative as an alternative drug of choice if the other agents do not cause differentiation). Previous results attained from cell survival assays (MTS metabolism assay, cell growth curve, and tumor cell clonogenic survival) as well as differentiation assays (accumulation of cytoplasmic lipid droplets and loss of Ki67 protein) showed that the combination of low concentrations of Aza or ATRA with CQ or HCQ decreased breast cancer cell survival and augmented the differentiation response more than either antimalarial alone. Task #1 is complete.

Task #2 of this project was to determine the optimum conditions for histone H4 hyperacetylation. To examine whether pretreatment with the demethylating agent, 5-Aza-dC (Aza), or the differentiating agent, ATRA, would alter the acetylation status of histones, western blot analyses were conducted to assess acetylated histone H3 and/or histone H4 and HDAC protein levels. As described previously, cell-free HDAC activity assay was conducted to show that ATRA alone is responsible for the direct inhibition of HDAC (Figure 1).

In addition, a non-radioactive ELISA assay containing PCAF as an active source of HAT enzyme was used to test whether the observed acetylation response is due to modulation of HAT activity (Figure 2). Interestingly, both ATRA and hydroxychloroquine caused direct HAT activation shown by increased acetylation of histone H3 and histone H4 substrate peptides. Hydroxychloroquine, augmented HAT activity more than ATRA alone; however, the combination of the drugs showed a more pronounced activation of HAT compared to basal levels. The hypothesis was that HCQ acts via HAT activation. The overall data suggest that direct HAT activation might contribute to both MCF-7 and MDA-MB-231 cell differentiation in response to the differentiation quinoline, hydroxychloroquine. On the other hand, ATRA seems to display dual roles by activating HAT as well as directly inhibiting HDAC. ATRA works thru the same mechanism of hydroxychloroquine by modulating HAT activity; therefore, an increase in HAT activity was seen upon combination treatment with both hydroxychloroquine and ATRA. However, ATRA acts via an independent mechanism by inhibiting HDAC enzymatic activity. Thereby, enhanced antiproliferative, differentiation, and histone acetylation responses are achieved during combination therapy with ATRA rather than the differentiating quinoline alone. Task #2 is complete.

Task #3 of this project was to develop a qualitative and quantitative assay using mass spectrometry for assessment of the overall histone acetylation profile, while Task #4 is to utilize this new assay using the most promising combination of agents. Since ATRA in combination with HCQ had the most significant inhibitory effect on clonogenic survival, and since clonogenic survival is the most sensitive test for predicting the responsiveness of a tumor to clinical treatment, the combination of ATRA with HCQ was assessed subsequently by mass spectrometry to measure its effect on histone H3 and histone H4 acetylation sites.

Histone H3 and histone H4 samples were resolved by SDS-PAGE and visualized using Coomassie Blue staining. The histone bands were excised and digested with the endoprotease, trypsin. The resulting tryptic peptides were analyzed by mass spectrometry to determine their individual mass values. Mass values for the specific peptide sequences of the N-terminal portion of histone H3 or histone H4 were attained by matching the measured masses with expected calculated mass values acquired from the International Protein Index (IPI) human database using Sequest Software. Acetylation sites were identified using a differential modification of 42 Daltons added to Lysine residues. The acetylation sites for the N-terminal portion of histone H3 were established showing 13 possible acetylation sites at Lys(K)-4, 9, 14, 18, 23, 27, 36, 37, 56, 64, 79, 115, and 122 (Figure 3) and 11 possible acetylation sites for histone H4 at Lys(K)-5, 8, 12, 16, 20, 31, 44, 59, 77, 79, and 91 (Figure 4A). This pattern was observed for n=2 experiments.

In figure 3, control cells treated with solvent (DMSO) only displayed acetylated Lysine residues at all possible sites except for Lys-4, and 9. Cells treated with hydroxychloroquine only showed acetylated Lysine residues at all possible sites except for Lys-4; however, cells pretreated with 1μM ATRA before hydroxychloroquine addition displayed acetylated Lysine residues at all possible sites including Lys-4. Cells treated with only TSA (positive control) also were acetylated at all possible Lysine sites. These results were consistent with the hypothesis of a "zip" model, whereby acetylation of histone H3 proceeds in the direction of from the N-terminal tail Lysine residues to Lys-4, and deacetylation proceeds in the reverse direction. In summary, hydroxychloroquine alone showed acetylation at Lys-9 sites compared to control cells. The modification of acetylation sites proceeded in the direction of acetylation to cover the remaining Lys-4 sites when cells were pretreated with 1μM ATRA.

In figure 4, control cells treated with solvent (DMSO) only displayed acetylated Lysine residues at all possible sites except for Lys-5, 8, and 12. Cells treated with hydroxychloroquine only showed acetylated Lysine residues at all possible sites except for Lys-5, and 8. Interestingly, cells with only TSA treatment (positive control) and cells pretreated with 1μM ATRA before hydroxychloroquine addition displayed acetylated Lysine residues at all possible sites (Figure 4A,B). These results were again consistent with the hypothesis of a "zip" model, whereby acetylation of histone H4 proceeds in the direction of from the N-terminal tail lysine residues to Lys-5, and deacetylation proceeds in the reverse direction. In summary, hydroxychloroquine alone showed modification at Lys-12 sites compared to control cells. The remaining acetylation sites, Lys-5, and 8 were modified when cells were pretreated with 1μM ATRA. Task #3 and Task #4 are complete.

Training Accomplishments

In addition to the above stated research accomplishments, the P.I. has made several training achievements during the July 1, 2004 through June 30, 2005 funded year. The P.I. successfully defended her dissertation in December of 2004. The P.I. also presented these findings at the Era of Hope meeting in June 2005.

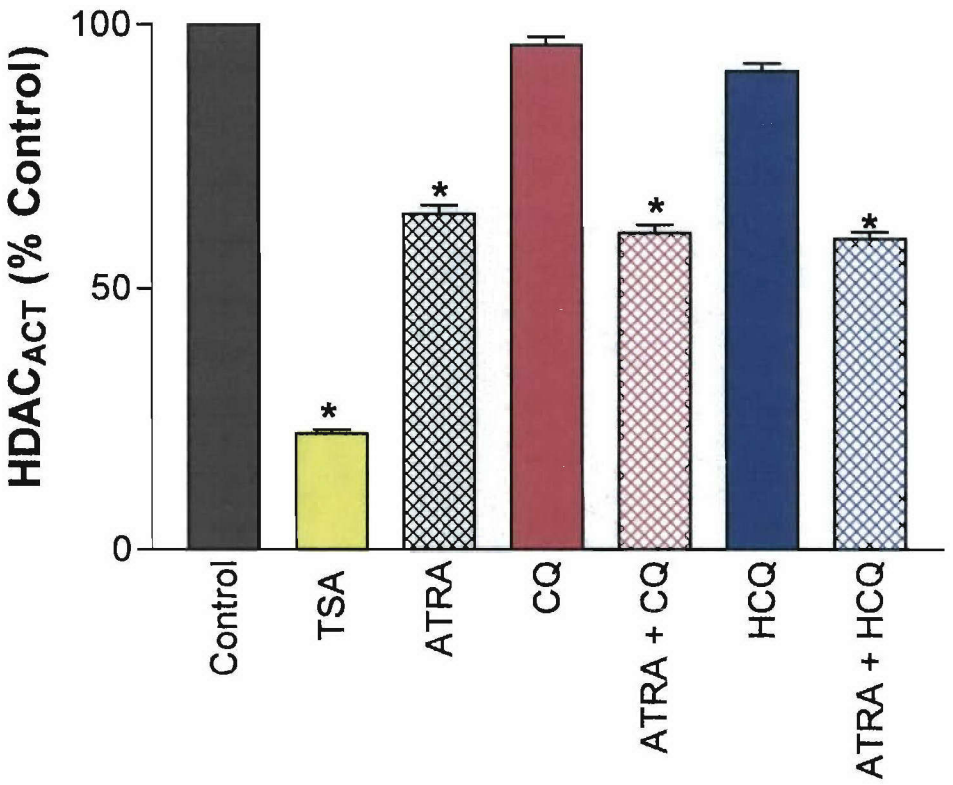


Figure 1. Histone Deacetylase (HDAC) Fluorescent Activity of Chloroquine or Hydroxychloroquine ± ATRA. Drugs were screened for the ability to directly inhibit histone deacetylase (HDAC) activity in HeLa cell nuclear extracts using the HDAC Fluorescent Activity Assay. TSA (35nM) was used as a positive control. Chloroquine (MCF-7 IC_{50}=33μM) or hydroxychloroquine (MCF-7 IC_{50}=57μM) ± 1μM ATRA were tested for HDAC activity. Data are the mean of n=3 experiments ± SEM performed in triplicates per treatment. Statistically significant differences from the control are indicated (*$p<0.05$).

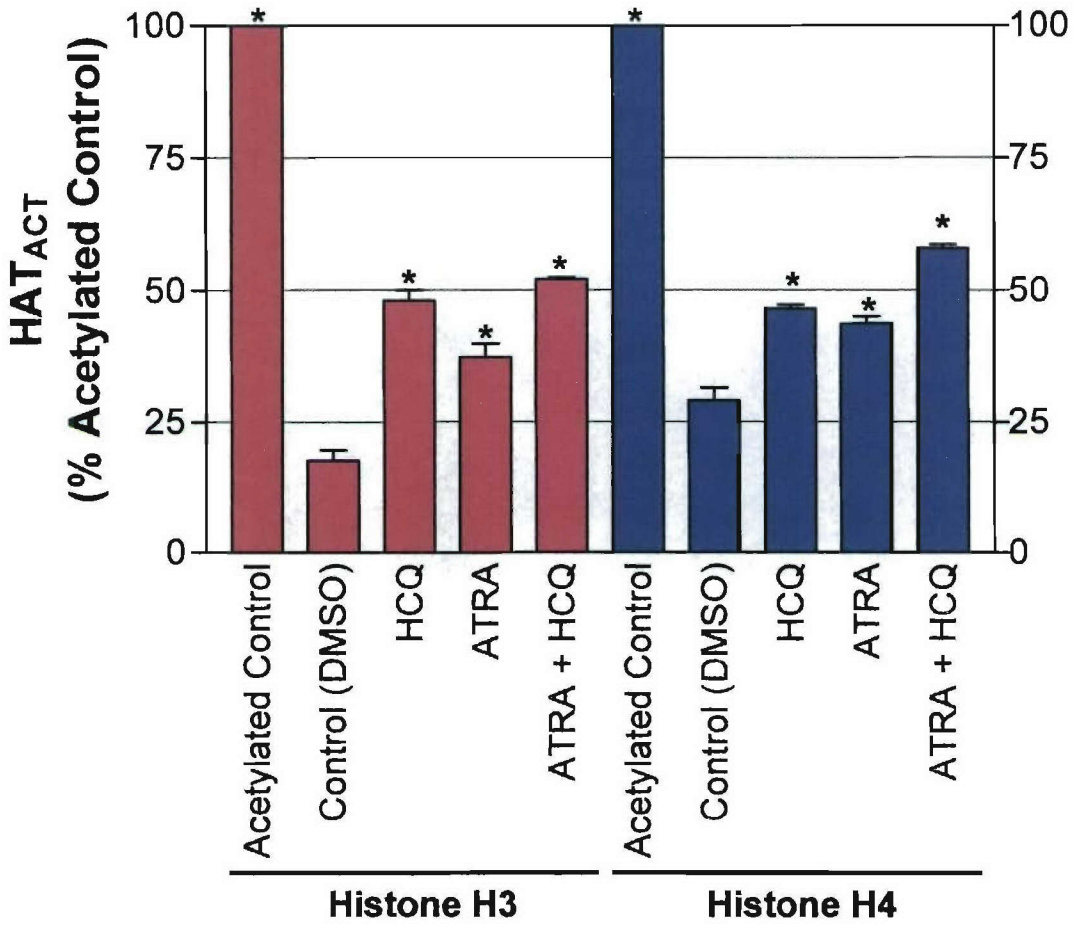


Figure 2. Histone Acetyltransferase (HAT) Activity of Hydroxychloroquine ± ATRA. Drugs were screened using an indirect ELISA Assay for the detection of acetyl residues on biotinylated Histone H3 or Histone H4 peptides using 75ng of recombinant PCAF, an active histone acetyltransferase enzyme, in the presence of 100μM Acetyl-CoA and HAT assay buffer. Acetylated Histone H3 or Histone H4 peptides were used as positive controls. Hydroxychloroquine (MCF-7 IC_{50}=57μM) ± 1μM ATRA were tested for HAT activity. Data are the mean of n=3 experiments ± SEM performed in triplicates per treatment. Statistically significant differences from control (DMSO) are indicated (*$p<0.05$).

Figure 3. Histone H3 acetylation status in MCF-7 cells following treatment with Hydroxychloroquine ± ATRA. $1x10^7$ MCF-7 cells were either pretreated with 1μM ATRA or not for 24 hours. Cells were then treated with solvent (DMSO; control), trichostatin A (TSA, 300nM), or hydroxychloroquine (CQ, IC_{50}=57μM) for another 12 hours. Total histone proteins were isolated using acid-extraction protocol from Upstate. 20μg of purified histone proteins were resolved on a 15% acrylamide gel and confirmed by Coomassie Blue staining. Histone H3 bands were excised, trypsin digested, and separated using reverse-phase HPLC with C18 column. Samples were analyzed on a ThermoFinnigan LCQ Deca XP Plus ion trap. MS/MS spectra were searched against International Protein Index (IPI) human database using Sequest Software. Acetylation sites were identified using a differential modification of 42 Daltons added to Lysine residues.

Acetylated Histone H3

Control (DMSO)

Hydroxychloroquine

ATRA + Hydroxychloroquine

TSA

Figure 4A,B. Histone H4 acetylation status in MCF-7 cells following treatment with Hydroxychloroquine ± ATRA. A.) $1x10^7$ MCF-7 cells were either pretreated with 1μM ATRA or not for 24 hours. Cells were then treated with solvent (DMSO; control), trichostatin A (TSA, 300nM), or hydroxychloroquine (HCQ, IC_{50}=57μM) for another 24 hours. Total histone proteins were isolated using acid-extraction protocol from Upstate. 20μg of purified histone proteins were resolved on a 15% acrylamide gel and confirmed by Coomassie Blue staining. Histone H4 bands were excised, trypsin digested, and separated using reverse-phase HPLC with C18 column. Samples were analyzed on a ThermoFinnigan LCQ Deca XP Plus ion trap. MS/MS spectra were searched against International Protein Index (IPI) human database using Sequest Software. Acetylation sites were identified using a differential modification of 42 Daltons added to Lysine residues. **B.)** MS/MS spectra of a histone H4 peptide sequence in cells pretreated with 1μM ATRA before hydroxychloroquine (HCQ, IC_{50}=57μM) addition.

Figure 4A.

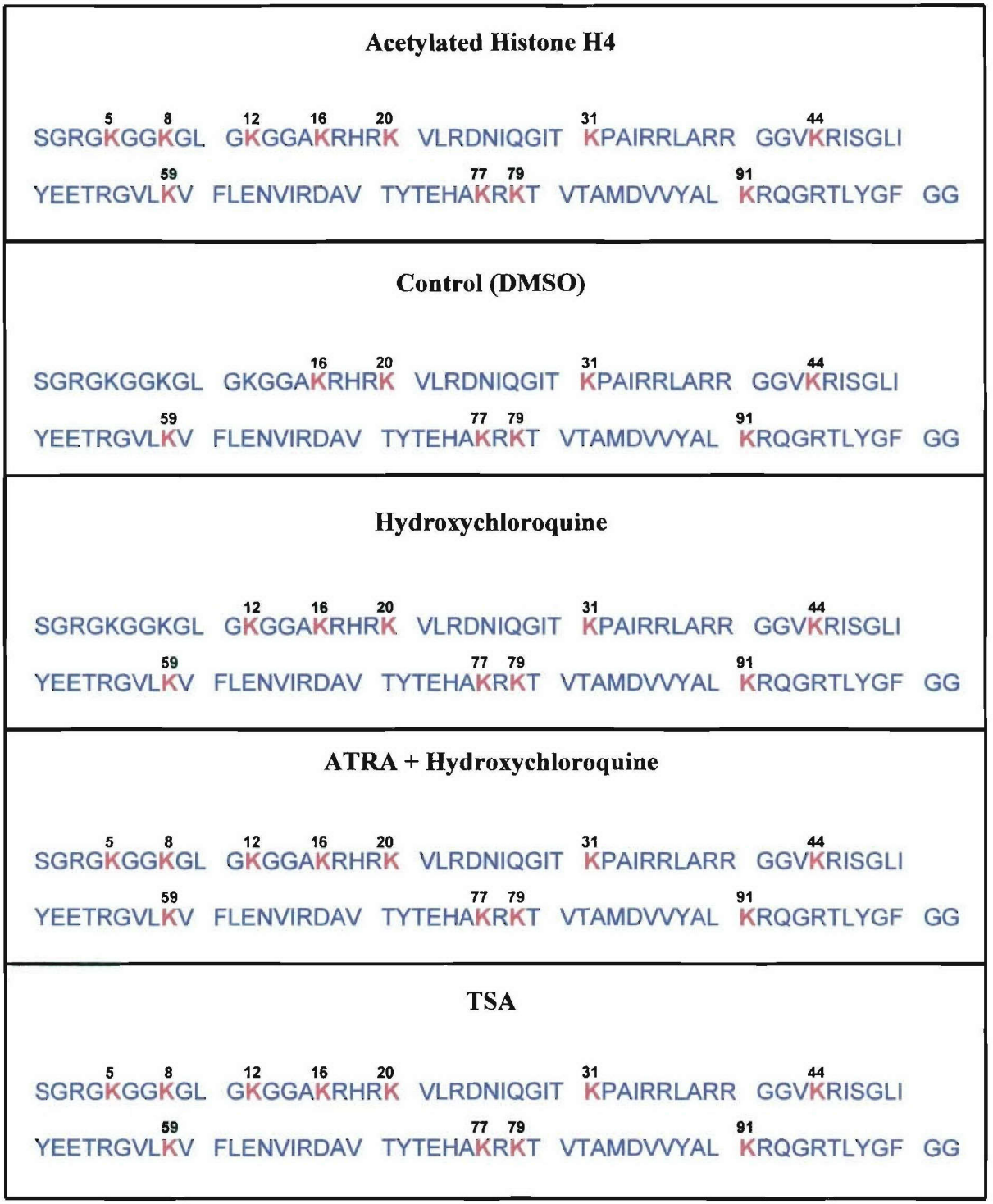
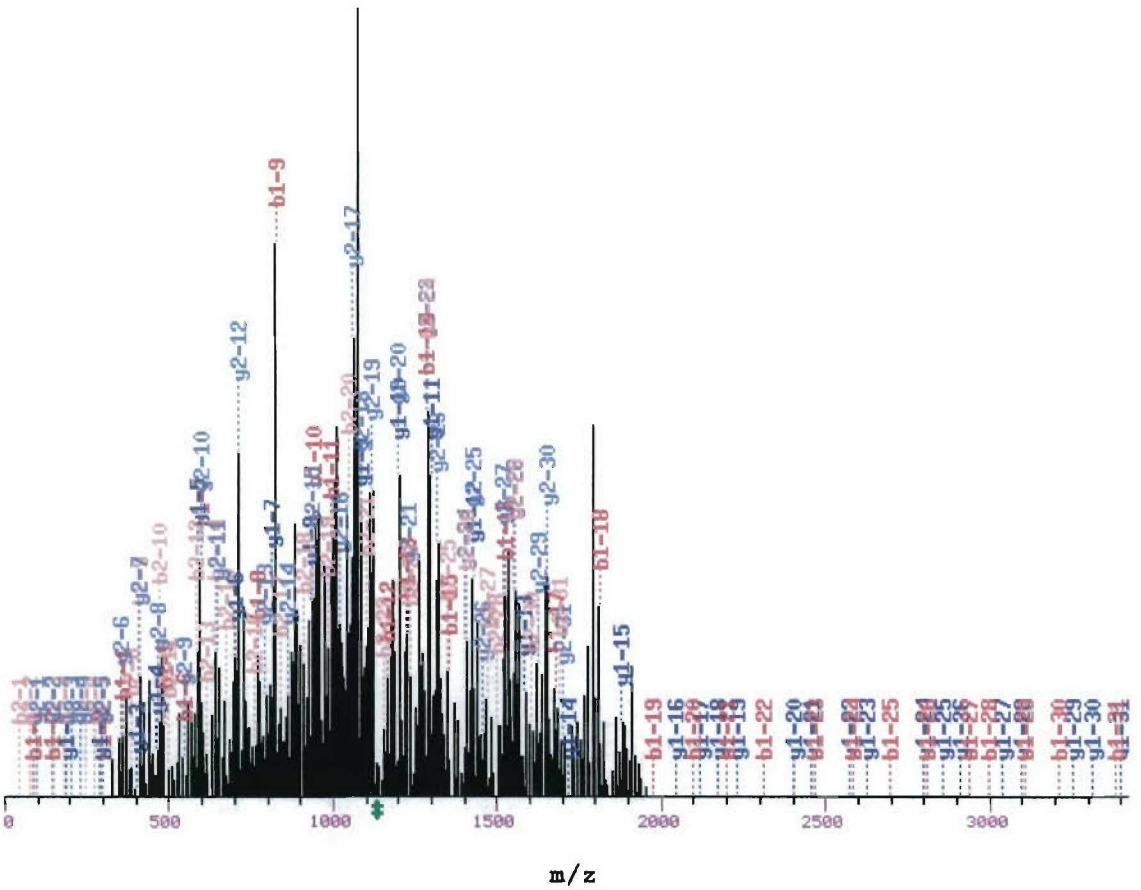

Acetylated Histone H4
5 8 12 16 20 31 44
SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
59 77 79 91
YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF GG
Control (DMSO)
16 20 31 44
SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
59 77 79 91
YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF GG
Hydroxychloroquine
12 16 20 31 44
SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
59 77 79 91
YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF GG
ATRA + Hydroxychloroquine
5 8 12 16 20 31 44
SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
59 77 79 91
YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF GG
TSA
5 8 12 16 20 31 44
SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
59 77 79 91
YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF GG

Figure 4B. ATRA + Hydroxychloroquine

Seq	#	b	y	(+1)
S	1	88.0	3397.9	31
G	2	145.1	3310.9	30
R	3	301.2	3253.8	29
G	4	358.2	3097.7	28
K	5	486.3	3040.7	27
G	6	543.3	2912.6	26
G	7	600.3	2855.6	25
*K	8	770.4	2798.6	24
G	9	827.4	2628.5	23
L	10	940.5	2571.5	22
G	11	997.5	2458.4	21
*K	12	1167.6	2401.4	20
G	13	1224.7	2231.3	19
G	14	1281.7	2174.2	18
A	15	1352.7	2117.2	17
*K	16	1522.8	2046.2	16

Seq	#	b	y	(+1)
R	17	1678.9	1876.1	15
H	18	1816.0	1720.0	14
R	19	1972.1	1582.9	13
K	20	2100.2	1426.8	12
V	21	2199.2	1298.7	11
L	22	2312.3	1199.7	10
R	23	2468.4	1086.6	9
D	24	2583.4	930.5	8
N	25	2697.5	815.5	7
I	26	2810.6	701.4	6
Q	27	2938.6	588.3	5
G	28	2995.7	460.3	4
I	29	3108.7	403.2	3
T	30	3209.8	290.2	2
*K	31	3379.9	189.1	1



Key Research Accomplishments:

- Conditions for cellular differentiation optimized. (Task #1)
- Optimization of histone isolation and Western blotting conditions. (Task #2)
- Optimization of qualitative assay using mass spectrometry. (Task #3)
- Analyzed most promising agents using mass spectrometry. (Task #4)

Reportable Outcomes:

Martirosyan, A.R., Rahim-Bata, R., Freeman, A.B., Clarke, C.D., Strobl, J.S. (2004) **Identification of differentiation-inducing quinolines as experimental breast cancer agents in the MCF-7 human breast cancer cell model.** *Biochemical Pharmacology* 68(9):1729-1738 (copy enclosed).

Rahim-Bata, R. and Strobl, J.S. (2005) Sensitization of human breast cancer cells to the antitumor activity of differentiation-inducing quinolines upon combination treatment with retinoic acid. (in preparation).

Abstracts:

Strobl, J.S. and Rahim-Bata, R. (2005) Distinct N-terminal histone H3/H4 modifications during MCF-7 mammary tumor cell differentiation by all-*trans* retinoic acid and hydroxychloroquine. (*American Association for Cancer Research, Abstract #2745*).

Rahim-Bata, R. and Strobl, J.S. (2005) Novel drug combination using retinoic acid and differentiation-inducing quinolines sensitize human breast cancer cells to enhanced antitumor activity. (*U.S. Army Medical Research and Materiel Command: Era of Hope, Department of Defense Breast Cancer Research*).

Conclusions:

The proposed work focused on using two promising antitumor antimalarials in combination with the demethylating agent, 5-Aza-dC, or with the differentiating agent, ATRA in order to lower the threshold for chemotherapy-induced cell death in breast cancer cells by augmenting their differentiation response. Cell survival, cellular differentiation, histone H3 and/or histone H4 acetylation status, and HDAC protein and activity were measured in order to optimize conditions for a new and highly sensitive assay for histone acetylation by mass spectrometry. This new approach illustrated the specific lysine sites that get modified (acetylated/deacetylated) by the most promising combination of chemotherapeutic agents to generate an overall histone acetylation profile.

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Differentiation-inducing quinolines as experimental breast cancer agents in the MCF-7 human breast cancer cell model

Anna R. Martirosyan[a,1], Rayhana Rahim-Bata[a,1], Andrew B. Freeman[a], Charles D. Clarke[a], Rachael L. Howard[a], Jeannine S. Strobl[a,b,*]

[a]*Department of Biochemistry & Molecular Pharmacology, West Virginia University, Morgantown, WV 26506, USA*
[b]*Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV 26506, USA*

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Abstract

The purpose of this work is to develop agents for cancer differentiation therapy. We showed that five antiproliferative quinoline compounds in the National Cancer Institute database stimulated cell differentiation at growth inhibitory concentrations (3–14 μM) in MCF-7 human breast tumor cells in vitro. The differentiation-inducing quinolines caused lipid droplet accumulation, a phenotypic marker of differentiation, loss of Ki67 antigen expression, a cell cycle marker indicative of exit into G_0, and reduced protein levels of the G_1 – S transcription factor, E2F1. The antimalarial quinolines, chloroquine, hydroxychloroquine and quinidine had similar effects in MCF-7 cells, but were 3–10 times less potent than the NSC compounds. NSC3852 and NSC86371 inhibited histone deacetylase (HDAC) activity in vitro and caused DNA damage and apoptosis in MCF-7 cells, consistent with their differentiation and antiproliferative activities. However, the HDAC assay results showed that for other compounds, direct HDAC enzyme inhibition was not required for differentiation activity. E2F1 protein was suppressed by all differentiation quinolines, but not by non-differentiating analogs, quinoline and primaquine. At equivalent antiproliferative concentrations, NSC69603 caused the greatest decrease in E2F1 protein (90%) followed by antimalarials quinidine and hydroxychloroquine. NSC69603 did not cause DNA damage. The other NSC compounds caused DNA damage and apoptosis and reduced E2F1 levels. The physicochemical properties of NSC3852, NSC69603, NSC86371, and NSC305819 predicted they are drug candidates suitable for development as experimental breast tumor cell differentiation agents. We conclude DNA damage and reductions in E2F1 protein are mechanistically important to the differentiation and antiproliferative activities of these quinoline drug candidates.

Keywords: Breast cancer; Differentiation therapy; E2F; MCF-7; Novel antitumor agents; Quinolines

Abbreviations: HDAC, histone deacetylase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PAMPA, parallel artificial membrane permeation assay; Rb, retinoblastoma protein; Amodiaquin, 4-[(7-chloro-4-quinolinyl)amino]-2-[diethylamino)-methyl]phenol; Chloroquine, N4-(7-chloro-4-quinolinyl)-*N*1,*N*1-diethyl-1,4-pentanediamine; Halofantrine, 1,3-dichloro-α-[2-(dibutylamino)ethyl]-6-(trifluoromethyl)-9-phenanthrenemethanol; Hydroxychloroquine , 2-[[4-[(7-chloro-4-quinolinyl)amino]pentyl]ethylamino]ethanol; Mefloquine, piperidinyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol); Quinidine , (9*S*)-6′-methoxycinchonan-9-ol; Quinine, (8-α,9*R*)-6′-methoxycinchonan-9-ol; Primaquine, *N*4-(6-methoxy-8-quinolinyl)-1,4-pentanediamine; NSC10010, *N*,*N*-bis(2-methyl-6-methoxy-4-quinaldyl)-1,9-diamine-nonane dihydrochloride hydrate; NSC3852, 5-nitroso-8-quinolinol; NSC69603, 4-(2-(2,5-dimethoxyphenyl)vinyl)quinoline; NSC86371, 2-[2-(8-hydroxy-5-methyl-7-quinolyl)vinyl]-1,6-dimethyl iodide; NSC305819, 1-(2,8-bis(trifluoromethyl)-4-quinolinyl)-3-(tertbutylamino)-1-propanol

* Corresponding author. Present address: Virginia College of Osteopathic Medicine, Virginia Tech Corporate Research Center, Blacksburg, VA, USA. Tel.: +1 540 231 1463.
E-mail address: jstrobl@vcom.vt.edu (J.S. Strobl).
[1] The first two authors contributed equally to this work.

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Differentiation therapy involves the use of drugs that modulate gene expression to control tumor growth. Interest in cancer differentiation agents as an alternative or supplement to traditional cancer chemotherapeutic drugs has increased in recent years. Histone deacetylases (HDACs) are the target of a number of tumor differentiation agents [1–3]. Catalytic HDAC inhibitors elicit a common profile of responses in tumor cells including histone hyperacetylation, elevated expression of the cyclin-dependent kinase inhibitor, p21/WAF-1, cell cycle arrest and apoptosis. Of note, many HDAC enzyme inhibitors that cause differentiation and apoptosis in human tumor cells were first identified as antifungal or antiprotozoal agents [4–6].

We observed growth arrest and differentiation in human breast tumor cells after treatment with the natural cinchona tree bark antimalarial, quinidine [7]. Quinidine-treated

MCF-7 cells displayed an enlarged cytoplasm filled with lipid-droplets. Cells were morphologically indistinguishable from cells treated with the histone deacetylase inhibitor trichostatin A, and comparable changes in the G_1 cell cycle protein profile including induction of p21/WAF, hypophosphorylation of retinoblastoma protein (pRb) and histone H4 hyperacetylation were observed. However, quinidine did not inhibit histone deacetylase catalytic activity suggesting that differentiation agents can engage alternative biochemical pathways. On this basis, we screened a panel of antimalarials and breast tumor cell antiproliferative quinolines from the NCI database for in vitro breast tumor differentiation activity to identify new experimental differentiation agents. Five differentiation quinolines are described which differ in their effects on histone deacetylase, DNA and apoptosis.

1. Materials and methods

1.1. Chemicals

Quinidine, chloroquine, primaquine, amodiaquin, quinine, quinoline, quinolinic acid and trichostatin A (TSA) were purchased from Sigma Chemical Company . Hydroxychloroquine and the NSC investigational compounds were gifts. Water soluble compounds were prepared as 1000× stocks and diluted directly into the assays. An equal volume of water was used as the solvent control. All other compounds were prepared as 1000–10,000× stock solutions in dimethyl sulfoxide and were diluted in tissue culture medium or assay buffer as appropriate; the final concentration of the solvent was $\leq$0.1% in all experiments, and an equal concentration of dimethyl sulfoxide was added to control groups. All other reagents were purchased from Sigma or Fisher Scientific.

1.2. Antibodies

Ki67 antibodies were purchased from Dako Corporation; E2F-1 and β-catenin antibodies were obtained from Santa Cruz Biotechnology, Inc. Peroxidase-conjugated secondary antibodies were used at dilutions recommended by the suppliers and reactions were visualized using Super Signal (Pierce).

1.3. Cell culture

The MCF-7 human breast cancer cell line (passages 43–60) was grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 40 μg/ml gentamicin at 37 °C in a humidified atmosphere of 6% CO_2/94% air. Cells were passaged weekly. Experiments were conducted with cell populations sub-cultured from confluent flasks into medium containing 5% serum. MCF10A (ATCC) were grown in Mammary epithelial growth medium.

1.4. MTS assay

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega) is metabolized by mitochondria in living cells to a colored formazan product. MCF-7 cells were plated in 96-well plates and incubated for 24 h without drugs. Serial dilutions of each compound or the solvent alone were added to the wells in triplicate and cells were incubated for 48 h before addition of the MTS substrate. Absorbance (490 nm) was determined after a 2 h incubation at 37 °C with MTS. Concentration–response data were fit by nonlinear regression using PrismGraphPad software to obtain the MTS IC_{50} values.

1.5. Cell differentiation end-points

Oil Red O: Cells were plated onto sterile glass coverslips placed into 35 mm^2 tissue culture dishes, and MTS IC_{50} levels of compounds were added for 48 h. To measure cytoplasmic lipid droplet accumulation cells were fixed, stained with Oil Red O and counterstained with Mayer's hematoxylin [8]. The threshold used to define a positive Oil Red O response was $\geq$10% of cells containing at least 1–10 lipid droplets/cell. For most compounds, 1 μM was the lowest concentration tested. Trichostatin A was used as the positive control for these assays, and 10 nM TSA caused a positive Oil Red O response in MCF-7 cells.

Ki67 index: Immunocytochemical detection of the cell cycle marker, nuclear Ki67, was performed as described previously [9]. The Ki67 index was calculated using the formula:

$$\frac{\%\ \text{Ki67 negative cells in treated cells}}{\%\ \text{Ki67 negative cells in control cells}}.$$

E2F-1 protein: E2F-1 protein levels in whole cell extracts were measured by Western blot analysis 48 h following addition of solvent or experimental compound (MTS IC_{50} levels) to the cell culture medium. E2F-1 chemiluminescent signals were captured using autoradiography and densitometric spot analysis with automatic background subtraction of the signals was performed using a FluorChem instrument (Alpha Innotech, San Leandro, CA). The E2F1 signals were normalized to the β-catenin signal [8] because previous studies showed that quinolines did not alter the levels of β-catenin.

1.6. HDAC assay

Compounds were screened for direct HDAC inhibition using HeLa cell extracts and a commercial assay kit (HDAC Fluorescence Activity Assay, BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA). MTS IC_{50} levels of each compound were added to cell extracts diluted in reaction buffer. Reactions were initiated by the addition of substrate (40 μM final concentration), and were con-

ducted for 10 min at 37 °C in triplicate wells of a 96-well plate. Product was quantified using 360 nm excitation and 460 nm emission (Cytofluor4000, PerSeptive Biosystems, Inc., Framingham, MA). Trichostatin A was used as the positive control in these assays; concentration–response experiments showed that HDAC activity in HeLa cell extracts was inhibited by trichostatin A with an IC_{50} value of 8.5 nM. Compounds active as HDAC inhibitors in HeLa cell extracts were retested in MCF-7 nuclear extracts prepared using the method of Dignam et al. [10].

1.7. Apoptosis determinations

The release of nucleosomal fragments into the cytoplasm of apoptotic cells was quantified using the Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science). The nucleosome-enrichment fraction was calculated as the ratio of the absorbance (405 nm) of treated cultures/solvent exposed cultures. The positive control, 30 μM etoposide, showed a nucleosome-enrichment fraction of 2.7.

1.8. Comet assay

MCF-7 cells were exposed to MTS IC_{50} levels of compounds or solvent alone for 24 h, then harvested and resuspended in ice-cold PBS (1.5×10^5 cells/ml). Fifty microliters of the cell suspension and 500 μl of 42 °C low melting point agarose were mixed, and 75 μl of the cell suspension spread over a Comet slide (Trevigen). Slides were processed in alkali solution as per the instructions of the manufacturer (Trevigen) and electrophoresed at 4 °C. DNA Comets were visualized after staining with SYBR green using a Nikon Eclipse TS100 microscope and FITC-cube at 640×. In cells with DNA damage, DNA fragments move from the cell nucleus (Comet head) into a tail-like structure. The Comet images were analyzed using the LAI Automated Comet Assay Analysis System (Loats Associates, Inc.). DNA damage was quantified by the tail moment = %DNA × distance traveled.

1.9. Parallel artificial membrane permeation assay (PAMPA)

Artificial membranes were prepared by treating Multi-Screen-IP PAMPA filter plates (Millipore Corp.) with a 1% (w/v) solution of lecithin in dodecane (5 μl/96-well filter). Acceptor and donor wells separated by the lipid membrane contained 5% DMSO in PBS, pH 7.4. As a measure of the ability of the test compounds to undergo intestinal absorption, each compound was added to the donor wells in duplicate at a final concentration of 500 μM. Incubations were performed at room temperature for 24 or 48 h, and then the contents of the acceptor and donor wells were transferred to UV transparent half-volume Corning 96-well plates (Fisher Scientific). The concentration of the test compounds in the donor well and acceptor wells was quantified by UV spectrophotometry using a SpectraMax 250 (Molecular Devices Corporation) and molar extinction coefficients of each compound.

1.10. Statistical analysis

Data were analyzed for statistical significance using a one-way ANOVA followed by Dunnett's *t*-test to compare treated groups to the appropriate solvent control group. The level of statistical significance used was $P < 0.05$.

2. Results

2.1. Cell differentiation activity

All of the compounds examined in the differentiation assays were antiproliferative in MCF-7 cells. Two antiproliferative measures, sulforhodamine blue (SRB) staining (GI_{50}) a growth arrest index reported by the NCI (http://dtp.nci.nih.gov or http://cactus.nci.nih.gov/ncidb2/), and inhibition of MTS metabolism (IC_{50}), independently determined under our experimental conditions are presented in Table 1. Our aim was to identify antiproliferatives capable of inducing cell differentiation. Differentiation activity was measured by accumulation of cytoplasmic lipid droplets and elevations in Ki67 index (Table 1). Lipid droplet accumulation was used as an end-point for differentiation activity because it occurs in MCF-7 cells induced to differentiate by treatment with Vitamin D analogs or histone deacetylase inhibitors [11,12]. Table 1 lists the lowest concentration (μM) of compound that induced the appearance of lipid droplets in MCF-7 cells during a 48 h incubation. Compounds that did not yield a positive Oil Red O response were not considered further. However, because the Oil Red O response lacked a mechanistic basis, we used a cell cycle marker, the Ki67 index as the more definitive indicator of differentiation activity. Ki67 is a nuclear protein in all actively cycling cells that is lost when cells exit the cell cycle and enter G_0 [13]. A Ki67 index = 1 reflects no change from control cells. The larger the Ki67 index, the greater the proportion of G_0 cells in the population. To compare differentiation activity among all the quinolines at equally antiproliferative concentrations, Ki67 assays were performed at a single concentration, the MTS IC_{50}.

Compounds **1**–**8** are antimalarials (Table 1 and Fig. 1). Among the antimalarials, quinolines with a 4-substitution were most active in these differentiation assays. Chloroquine (**1**), hydroxychloroquine (**2**), amodiaquin (**3**), quinidine (**4**) and quinine (**5**) were positive for lipid droplet accumulation and had Ki67 indeces >1 (Table 1). Mefloquine (**6**) was the only 4-substituted quinoline antimalarial that did not promote differentiation. The unsubstituted quinoline ring (**9**) was completely inactive. Halofantrine (**7**), an antimalarial phenanthrene, and primaquine (**8**), an

Table 1
Differentiation activity

#	NSC #	Name	Oil Red O (μM)	MTS IC50 (μM)	SRB GI50 (μM)	Ki67 index	HDAC activity (% control)
1	14050	Chloroquine	1	33	19	6.6*	91 ± 6
2	4375	Hydroxychloroquine	5	57	nd	3.6	94 ± 1
3	13453	Amodiaquin	10	7	nd	2.4	86 ± 4
4	10004	Quinidine	10	113	nd	5	100 ± 13
5	5362	Quinine	30	40	60	4.1	102 ± 14
6	157387	Mefloquine	Negative	3	nd	0.3	90 ± 2
7	305789	Halofantrine	5	11	10	1.4	81 ± 13
8	149765	Primaquine	2.5	3	16	1.1	95 ± 14
9		Quinoline	Negative	62	nd	0.4	96 ± 7
10	2039		15	8	2	1.6	95 ± 4
11	3852		10	10	2	7.2*	69 ± 6*
12		Quinolinic acid	Negative	28	nd	0.4	99 ± 6
13	69603		1	14	0.9	6*	111 ± 7
14	305819		1	7	13	5.8*	90 ± 17
15	124637		1	13	14	3.4	127 ± 14
16	4239		1.3	0.7	2	1.9	96 ± 4
17	10010		3.5	4	5.4	8.3*	94 ± 8
18	86371		1	6	nd	6*	65 ± 5*
19	86373		1	13	nd	4.7	97 ± 2
20	86372		2	0.2	0.1	0.8	91 ± 7
21	85700		1	7	7	4.1	97 ± 1
22	85701		0.5	4	4	2.7	90 ± 7
23	4238		0.7	0.4	2	1.9	109 ± 13

Numbers in the first column correspond to compound structures **1–23** (Figs. 1 and 2). For each compound, the lowest concentration that produced an Oil Red O response is shown. Antiproliferative activity is reported as the IC_{50} level for inhibition of mitochondrial metabolism of MTS following a 48h exposure to each compound. Data are the average IC_{50} of $n = 3$ experiments performed with 10 concentrations of each compound in triplicate. For comparison, the GI_{50} for sulforhodamine blue (SRB) staining reported on the NCI website is presented (nd: not determined). The Ki67 index is a measure of the fraction of G_0 cells in a population where a Ki67 index >1 indicates more G_0 cells in the population. Data shown are the average of at least $n = 3$ experiments in cells exposed for 48 h to the MTS IC_{50} level of each compound. Inhibition of histone deacetylase (HDAC) activity in vitro by IC_{50} levels of each compound is expressed as a percent of HDAC activity in solvent control groups. Data are the mean ± S.E.M. of at least $n = 3$ experiments/compound assayed in duplicate. Statistically significant differences in Ki67 index and HDAC activity ($P < 0.05$) are indicated by (*).

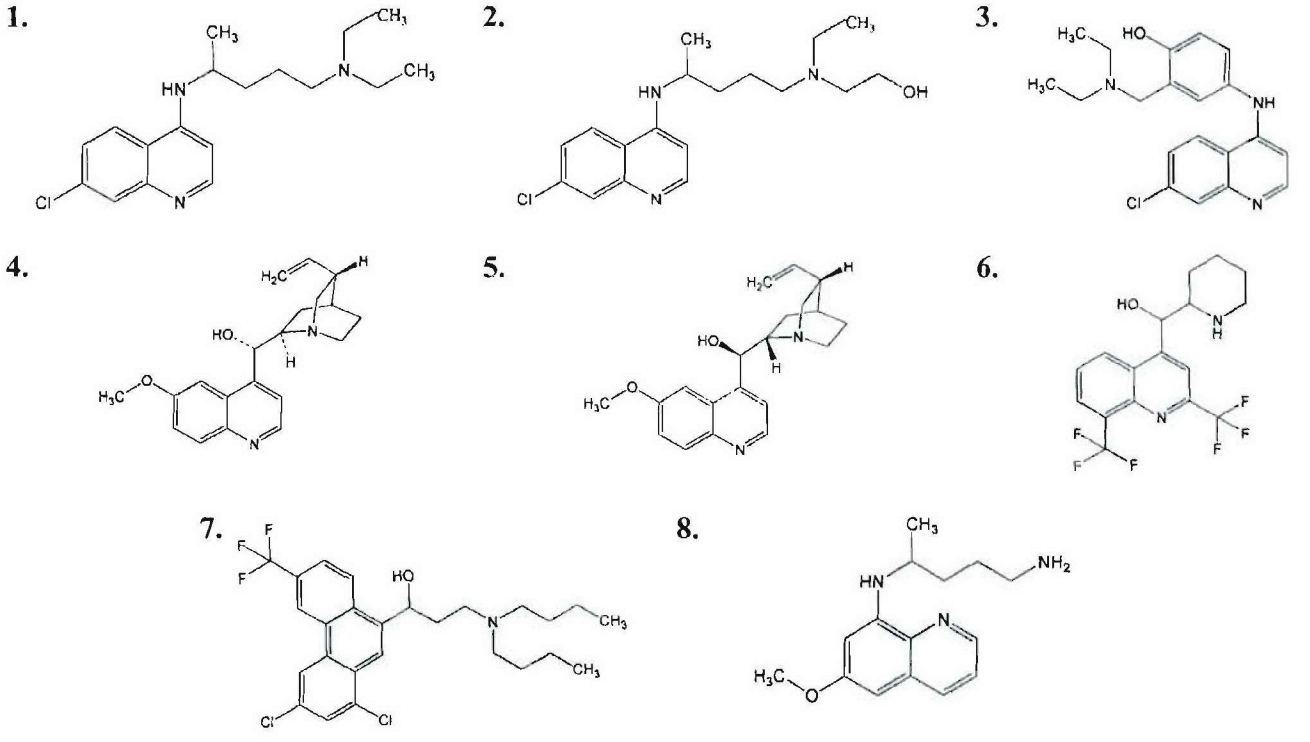

Fig. 1. Antimalarials. Chemical structures are presented and labeled numerically. Numeric labels correspond to NSC # and common names presented in Table 1.

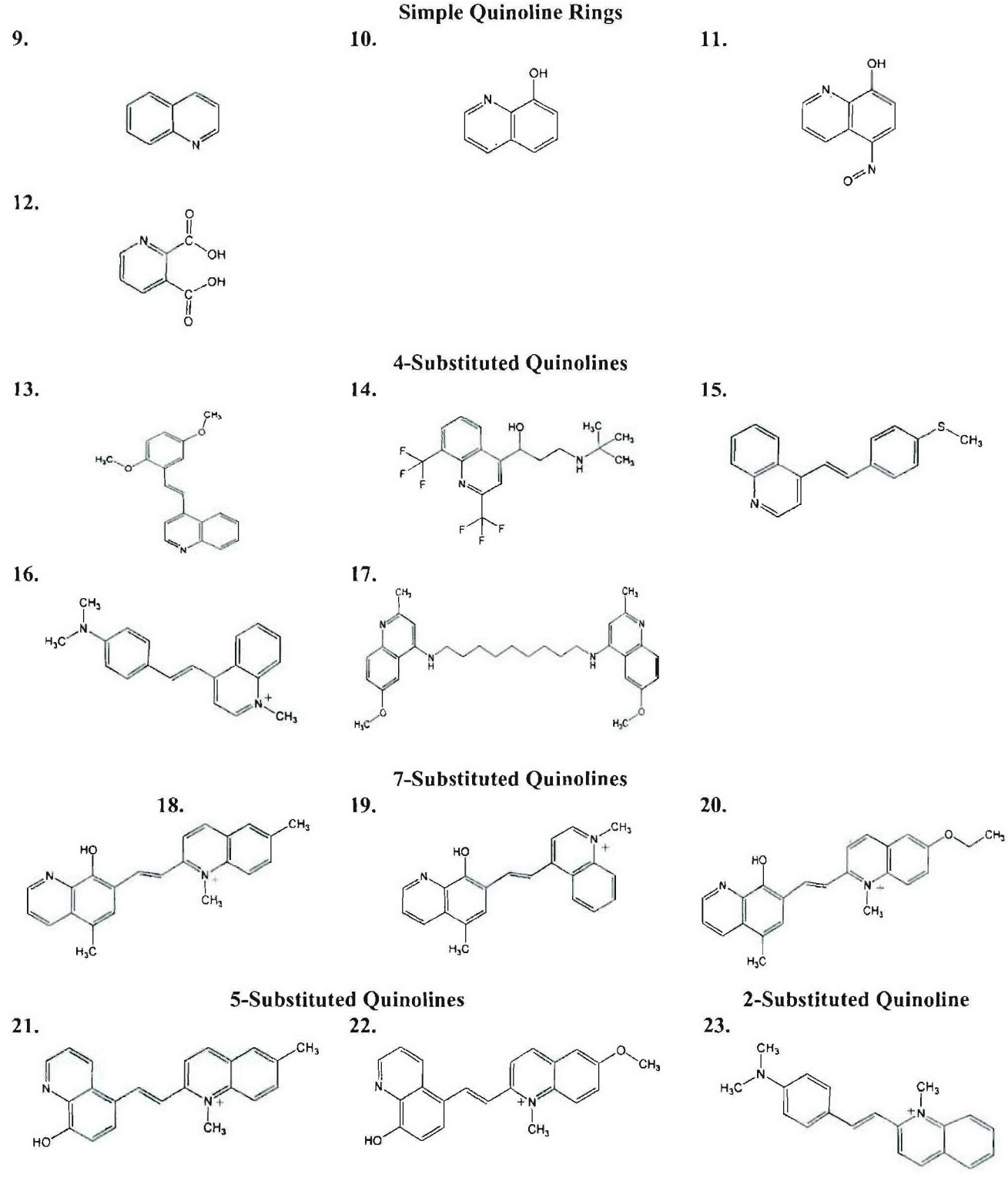

Fig. 2. NSC compounds. Chemical structures are presented and labeled numerically. Numeric labels correspond to NSC# presented in Table 1.

8-aminoalkyl substituted quinoline antimalarial gave mixed differentiation responses. Both compounds caused lipid droplet accumulation without effect upon the cell cycle marker, Ki67. We concluded antimalarial activity per se was not predictive of differentiation activity in this data set.

The antiproliferative quinoline NSC compounds shown in Fig. 2 were tested for differentiation activity in the same assays. Two minimally substituted quinolines (**10**, NSC2039 and **11**, NSC3852) and quinolinic acid (**12**) were included. Five 4-substituted quinolines, **13–17**, were tested: NSC69603 (**13**), NSC305819 (**14**), NSC124637

(**15**), NSC4239 (**16**) and NSC10010 (**17**). Three of these 4-substituted quinolines, similar to the antimalarial amodiaquin, contained phenyl substituents (**13** (NSC69603), **15** (NSC124637), **16** (NSC4239)) and one was a bisquinoline (**17** (NSC10010)). Compounds **13** (NSC69603), **17** (NSC10010), **21** (NSC86372), **22** (NSC85701) bore methoxy substituents as did the differentiating antimalarials, quinidine (**4**) and quinine (**5**). Quinidine and quinine differ in structure only as stereoisomers. Five NSC compounds and chloroquine (**1**) showed greater differentiation activity than quinidine (**4**) based upon the magnitude of the Ki67 index. The Ki67 index in MCF-7 cells after treatment with compounds **11** (NSC3852), **13** (NSC69603), **14** (NSC305819), **17** (NSC10010), **18** (NSC86371), and **1** (chloroquine) was statistically significantly greater than that of control cells. This group of compounds is referred to as the differentiation-inducing quinolines.

2.2. HDAC activity

All compounds were screened in HeLa cell nuclear extracts for the ability to directly inhibit human histone deacetylase activity (Table 1). Only compounds **11** (NSC3852) and **18** (NSC86371) significantly inhibited HDAC activity in HeLa cell extracts. Both compounds also statistically significantly inhibited HDAC in MCF-7 cell nuclear extracts, but in comparison with TSA, are weak HDAC inhibitors.The data show that HDAC inhibition might contribute to MCF-7 cell differentiation in response to compound **11** (NSC3852) and compound **18** (NSC86371). In contrast, direct inhibition of HDAC is unlikely to contribute to the differentiation response in MCF-7 cells by the other differentiation quinolines.

2.3. E2F-1 protein

The transcription factor E2F-1 is critical to the G1–S phase transition [14]. MCF-7 cells exposed to all of the differentiation quinolines for 48 h showed suppressed E2F-1 protein levels (Fig. 3). However, E2F-1 protein was unchanged in MCF-7 cells treated with compounds **8** (primaquine) and **9** (quinoline) that failed to induce differentiation. The data are consistent with the hypothesis that down-regulation of E2F-1 protein is characteristic of in vitro activity by differentiation quinolines, and facilitates the induction of differentiation.

2.4. Growth arrest and cell death

MCF-7 cell growth in the presence of the antimalarial compounds **1** (chloroquine), **2** (hydroxychloroquine), and **4** (quinidine), (Fig. 4A) and the differentiation-inducing

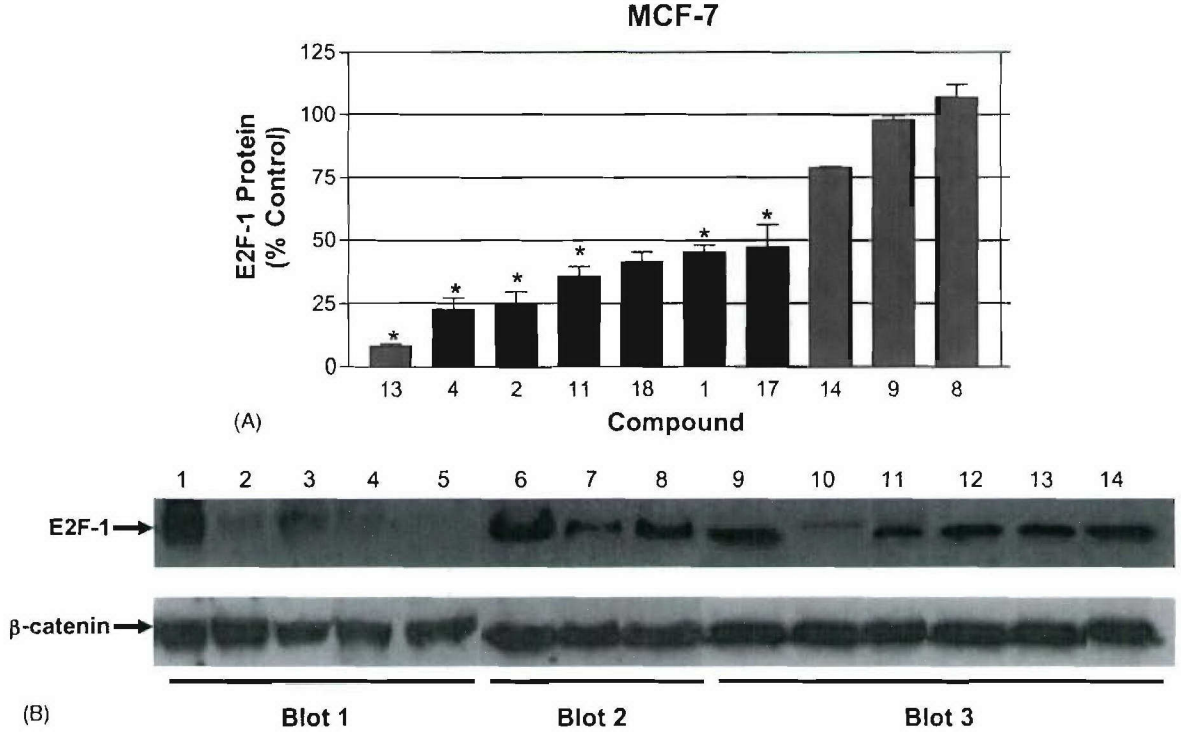

Fig. 3. Western blot analysis of E2F-1. MCF-7 cells were exposed to IC_{50} levels of compounds for 48 h. E2F-1 and β-catenin levels were detected using chemiluminescent autoradiography and quantified densitometrically using a FluorChem image analysis system. After normalization for the β-catenin levels in each lane, the E2F-1 protein levels were expressed as a percentage of the appropriate solvent control. Panel A is a summary of $n = 3$ or $n = 2$ experiments. Panel B is a composite of three Western blots showing the E2F-1 and β-catenin signals in control and treated cell extracts. Panel B, lanes 1–7 ($n = 3$) and lanes 8–14 ($n = 2$). Panel B: lane 1, 0.1% DMSO control; lane 2, **4** (quinidine); lane 3, **1** (chloroquine); lane 4, **2** (hydroxychloroquine); lane 5, **13** (NSC69603); lane 6, 0.1% DMSO control; lane 7, **11** (NSC3852); lane 8, **17** (NSC10010); lane 9, 0.1% DMSO control; lane 10, **18** (NSC86371); lane 11, **14** (NSC305819); lane 12, **8** (primaquine) (in water); lane 13, **9** (quinoline) (in water); lane 14, water control.

quinolines (Fig. 4B and C) were compared to confirm that MTS activity was a valid antiproliferative indicator. DNA damage was assessed on an individual cell basis using the Comet assay. The greater the DNA damage, the longer the Comet tail as quantified by the tail-moment (Fig. 5A). After 24 h, DNA damage by compounds **11** (NSC3852), **14** (NSC305819), **17** (NSC10010) and **18** (NSC86371) in MCF-7 cells was apparent. No damage to DNA by compound **1** (chloroquine) or **13** (NSC69603) was detectable. Similar results for all compounds were obtained after 48 h (data not shown). In MCF10A cells, very little DNA damage occurred except in response to compound **18** (NSC86371) (Fig. 5B). Short-term (48 h) growth experiments were conducted with MCF10A to assess the toxicity of the differentiation quinolines on non-transformed mammary epithelial cells (Fig. 6). MCF10A cell numbers were significantly reduced by all the differentiating quinolines. In comparison with the solvent treatment alone, compound **14** (NSC305819) was growth suppressive and less toxic than chloroquine to the normal cells. Compound **17** (NSC10010) was clearly toxic, and compounds **11** (NSC3852) and **18** (NSC86371) had intermediate effects on MCF10A cell numbers.

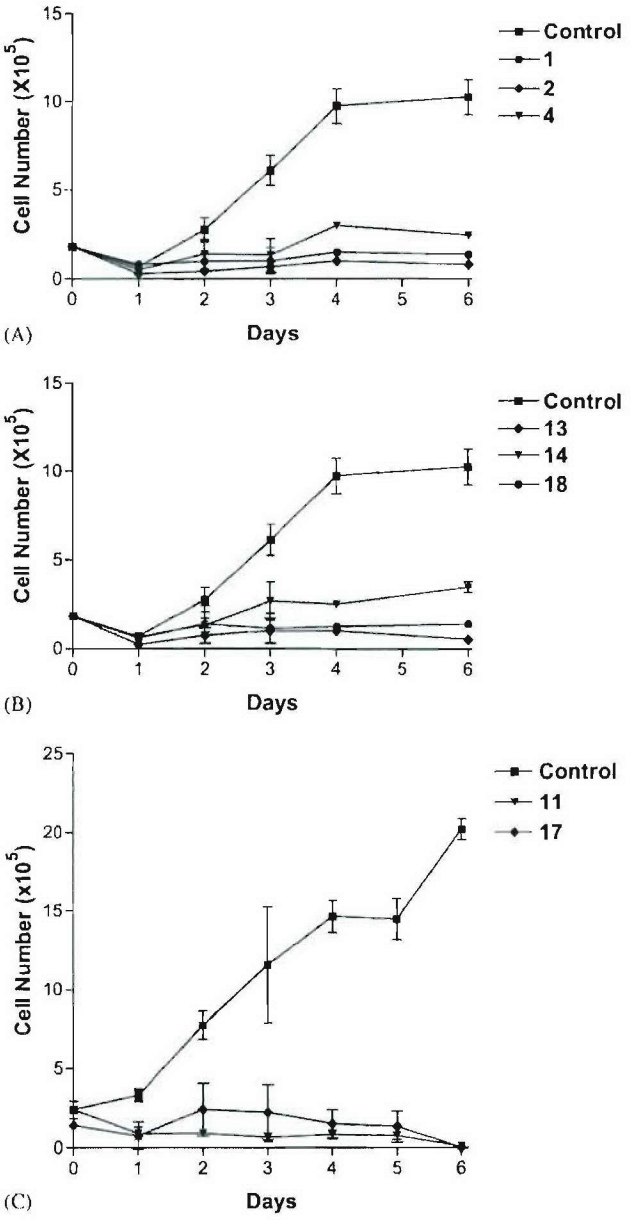

Fig. 4. MCF-7 growth inhibition. MCF-7 cells were grown in the presence of MTS IC_{50} levels of the quinoline differentiation compounds for 6 days. Data are the mean and range of duplicate determinations in two independent experiments.

MCF-7 cells undergo a slow apoptotic response that can be quantified using the nucleosome release ELISA. After 72 h, apoptosis was detected in response to all of the differentiation-inducing quinolines except compound **13** (NSC69603) (Fig. 7). In summary, all differentiation-inducing quinolines caused growth suppression in MCF-7 and MCF10A cells, but exhibited variable capacities to induce DNA damage and apoptosis. Compound **13** (NSC69603) was unique in its ability to cause equivalent growth suppression in the absence of DNA damage or apoptosis.

2.5. Pre-drug development screens

Drugs frequently exhibit a common set of physicochemical properties summarized by Lipinski et al. [15]. To facilitate drug development, these properties have been reported for the NSC compounds (http://cactus/nci.nih.gov/ncidb2/). As summarized in Table 2, compounds **11** (NSC3852), **13** (NSC69603), **14** (NSC305819) and **18** (NSC86371) meet the Lipinski rule of fives: a molecular weight $\leq$500, <5 hydrogen-bond donors, <10 hydrogen-bond acceptors, and a oil–water partition (log P) between −1 and +5 [15]. Compound **17** (NSC10010) had a log $P > 5$, but otherwise met the specifications defined by Lipinski et al. Compound **18** (NSC86371), a quarternary

Table 2
Predicted oral absorption of quinoline compounds

Compounds	Molecular weight (g/mol)	HIA >15%	PAMPA 24 h (μM)	PAMPA 48 h (μM)
1 (chloroquine)	319.9	1	25.8 ± 4.0*	51.1 ± 0.2*
11 (3852)	174.2	2	3.2 ± 1.8	7.1 ± 4.0
13 (69603)	291.3	1	10.2 ± 6.5	0
14 (305819)	394.4	1	59.1 ± 16*	64.8 ± 5.7*
17 (10010)	500.7	1	0	1.2 ± 1.2
18 (86371)	341.4	0	Nd	Nd

Chloroquine is the reference compound for a drug with very good oral absorption. HIA: human intestinal absorption predicted in silico using ADME/Tox Screen (Pharma Algorithms, North America, Toronto, Canada) where 0 = poor absorption predicted, 1 = non-restricted passive diffusion, and 2 = paracellular transport of small molecule or passive diffusion. PAMPA: parallel artificial membrane permeation assay. Concentrations of compounds in the acceptor wells (mean ± S.E.M. of $n = 3$ experiments performed in duplicate) after 24 or 48 h are shown. Statistically significant transfer of compounds across the membrane ($P < 0.05$) is indicated by (*). Nd: not determined.

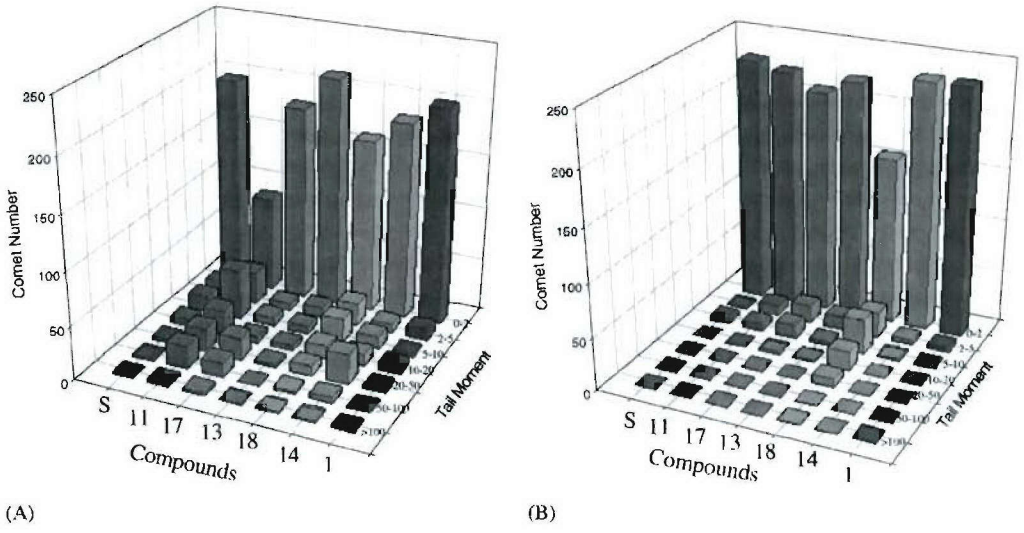

Fig. 5. Comet assay of DNA damage. (A) MCF-7 cells or (B) MCF10A cells were exposed to MTS IC_{50} levels of the quinoline differentiation compounds or chloroquine for 24 h. Damaged DNA released from the nuclear "head" and the Comet tail length was analyzed as a measure of the total DNA damage in individual cells. Eighty cells were analyzed/experimental condition. Low (0–2) Comet tail moments in control cells are indicative of minimal DNA breakage. Data shown are pooled results of three independent experiments in each cell line.

amine, has a fixed positive charge, indicative of poor oral absorption.

A rapid in vitro assay for transcellular permeation of drugs through artificial lipid membranes (PAMPA) is an experimental approach to predicting gastrointestinal drug absorption [16]. Gastrointestinal drug absorption properties predicted by PAMPA were Compound **14** (NSC305819) >> Compound **1** (chloroquine) > Compound **13** (NSC69603) >> Compound **11** (NSC3852) (Table 2). Compound **17** (NSC10010) showed poor permeability in our studies indicating that oral administration of this bisquinoline differentiation agent, as well as of Compound **18** (NSC86371) could mask antitumor activity in whole animal studies. In contrast, Compounds **11** (NSC3852), **13** (NSC69603), and **14** (NSC305819) are predicted to be active after oral administration.

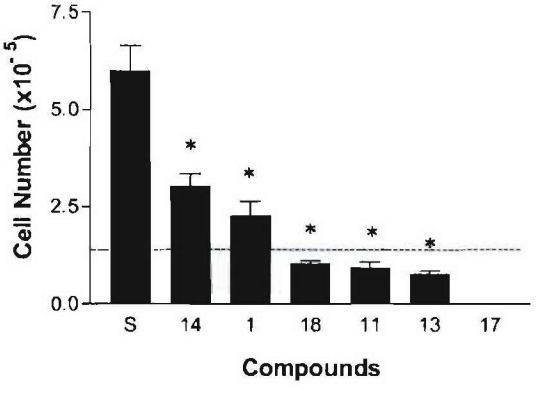

Fig. 6. MCF10A cell growth. MCF10A cells were incubated for 48 h in the presence of MCF-7 IC_{50} levels of differentiation quinolines, then harvested and viable cells counted. Data represent the mean ± range of three experiments. The dashed line indicates the number of cells plated at 0 time.

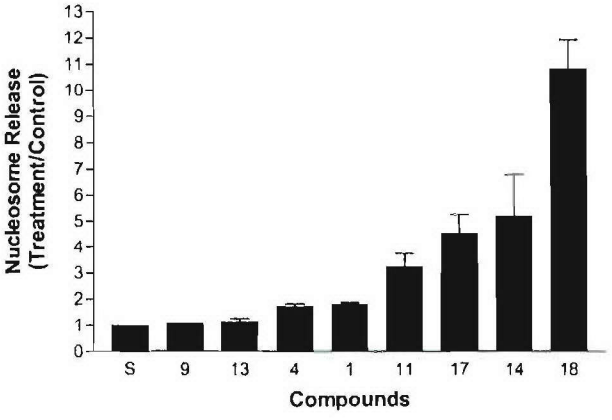

Fig. 7. Nucleosome ELISA for apoptosis. MCF-7 cells were treated with MTS IC_{50} levels of the quinoline differentiation compounds for 72 h. DNA–histone nucleosome complexes in the cell cytosol were quantified immunochemically. Apoptosis measured as nucleosome release is reported here as the ratio of treated/control values obtained in a single experiment performed in triplicate except for quinidine and chloroquine where the data represent $n = 3$ independent experiments performed in triplicate.

3. Discussion

Using the Ki67 index as a primary functional screen for agents that promote differentiation in MCF-7 cells, we identified six compounds for which differentiation contributed significantly to their antiproliferative activities. These compounds lacked a discernable common chemical structure other than the quinoline ring, however they shared an important functional capacity to suppress E2F1 protein levels. E2F1 is widely recognized as a cell cycle regulatory protein that fosters cell proliferation by stimulating the G1–S cell cycle transition. Moreover, E2F1

plays a modulatory role in cell differentiation. Down-regulation of E2F1 is a necessary pre-condition for cells to undergo differentiation, and E2F1 overexpression can act to suppress the differentiation response. Finally, under certain conditions, E2F1 is a pro-apoptotic protein. Because of its involvement in three critical cell decisions: growth, differentiation, and death, E2F1 is an attractive molecular target for drug discovery [17–19].

As a working model to explain the mechanism of action of the differentiation-inducing quinolines, we propose that strong suppression of E2F1 alone inhibits growth by preventing cell cycle progression and fosters differentiation by creating a permissive environment for cell differentiation. Moderate suppression of E2F1, in conjunction with induction of DNA damage, promotes differentiation through the activation of the p53–Rb pathway as well. Compound **13** (NSC69603) is an example of a quinoline differentiation compound that might act primarily through the suppression of E2F1. Compound **13** decreased total cell E2F1 levels by 90% but lacked DNA damage or apoptotic activity. Additional experiments are needed to test the hypothesis that E2F1 is the principal target of Compound **13**. Compounds **11** (NSC3852), **17** (NSC10010) and **18** (NSC86371) are examples of quinoline differentiation compounds that caused DNA damage and decreased E2F1 levels albeit more modestly (~50%). We know from previous work in MCF-7 cells that DNA damage causes Rb hypophosphorylation via increased p53 and the induction of the cyclin-dependent kinase inhibitor, p21/WAF-1 [9,20]. We propose that DNA damage and E2F1 suppression are important to the mechanism of action of Compounds **11, 17 and 18.** E2F1 complexes with hypophosphorylated Rb causing growth arrest through E2F1 sequestration. Cell differentiation is fostered by recruitment of co-repressor complexes to the promoter regions of E2F1 target genes by the E2F1–Rb complex [21,22].

Compounds **11** (NSC3852) and **18** (NSC86371) were identified as in vitro HDAC inhibitory quinolines at concentrations that induced differentiation in MCF-7 cells, 10 and 6 μM, respectively. Compound **11** (NSC3852) caused a similar decrement in E2F1 protein levels (60–70%) and also DNA damage. Hence, for these agents, three mechanisms potentially contributed to cell growth inhibition and differentiation: E2F1 suppression, DNA damage and HDAC inhibition. Although there is yet no direct evidence that gene specific changes in histone acetylation are required for the differentiation response in MCF-7 cells, comparison of the activity of a pair of compounds, Compound **11** (NSC3852) and Compound **10** (NSC2039, 8-quinolinol) provided indirect evidence for the involvement of HDAC. Compounds **10** and **11** differ only with respect to the presence of the nitroso substitution in Compound **11** (NSC3852). In the presence of the nitroso substitution, HDAC activity was inhibited ($P < 0.05$) and the Ki67 index was elevated ($P < 0.05$) while the 8-quinolinol analog, compound **10** (NSC2039), had no statistically significant effect on either HDAC activity or the Ki67 index. This pair of compounds will enable us to probe the mechanism of action of Compound **11** (NSC3852) in intact cells.

Compounds **11** (NSC3852), **13** (NSC69603) and **14** (NSC305819) exhibit drug-like chemical properties consistent with oral activity. Data available on the National Cancer Institute web site indicate that Compounds **11** and **13** sufficiently potent to exert antitumor activity in vivo. Compound **13** (NSC69603) demonstrated modest in vivo activity in two mouse mammary tumor models and the P388 leukemia model in mice. Compound **11** (NSC3852) showed activity against P388 leukemia growth in mice in NCI screening trials. There are no reported tests of in vivo antitumor activity of Compound **14** (NSC305819). The mechanism of action of Compound **14** in MCF-7 cells is the least clear as it caused a modest 25% decrease in E2F1 protein, minimal DNA damage, and no HDAC inhibition. However, Compound **14** (NSC305819) was the least toxic of all the differentiation-inducing quinolines in MCF10A cells and might have tumor selective activity. In conclusion, the differentiation-inducing quinolines comprise a new category of experimental breast cancer differentiation agents. Their use as lead compounds will complement ongoing efforts to develop differentiation therapies for breast cancer.

Acknowledgments

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